



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/02, 37/54, 37/62 C07H 15/12, 17/00 C07K 3/06, 3/08, 13/00 C12N 5/00, 9/70, 9/72 C12N 9/96, 15/00		A1	(11) International Publication Number: WO 92/19279
			(43) International Publication Date: 12 November 1992 (12.11.92)
(21) International Application Number:	PCT/US92/03960		(74) Agent: GUBINSKY, Louis; Sughrue, Mion, Zinn, Meck-peak & Seas, 2100 Pennsylvania Avenue, N.W., Suite 800, Washington, DC 20037-3202 (US).
(22) International Filing Date:	11 May 1992 (11.05.92)		
(30) Priority data:	697,364 9 May 1991 (09.05.91)	US	(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.
(71) Applicant (for all designated States except US):	BOARD OF REGENTS OF THE UNIVERSITY OF WASHINGTON [US/US]; Seattle, WA 98195 (US).		
(72) Inventors; and			Published
(75) Inventors/Applicants (for US only) :	TAIT, Jonathan [US/US]; 13716 39th Avenue Northeast, Seattle, WA 98125 (US). FUJIKAWA, Kazuo [JP/US]; 5016 Harold Place Northeast, Seattle, WA 98105 (US).		With international search report.

(54) Title: PHOSPHOLIPID-TARGETED THROMBOLYTIC AGENTS**(57) Abstract**

Conjugates with an affinity for phospholipids are disclosed. The conjugates comprise a first compound having affinity for phospholipids, with a binding constant that is not greater than about 10₋₇ M and a second compound that lyses thrombi or is a precursor of a compound that lyses thrombi.

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Bahamas	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

PHOSPHOLIPID-TARGETED THROMBOLYTIC AGENTS

Portions of the research described herein were supported in part by grants awarded by the National Institute of Health. The government has certain 5 rights in the invention.

FIELD OF THE INVENTION

The invention relates to conjugates and pharmaceutical compositions with affinity for phospholipids and capability of lysing fibrin 10 clots. The invention also relates to methods and therapeutic regimens for treating disorders relating to fibrin clots.

BACKGROUND OF THE INVENTION

Fibrin deposits form at sites of vascular 15 injury including ruptured atherosclerotic plaques. The initial event leading to fibrin formation is the activation of the extrinsic coagulation pathway which is triggered by the contact of tissue factor with circulating factor VII/VIIa. Tissue factor is 20 a membrane-bound regulatory protein present outside blood vessels as well as in atherosclerotic plaques. When tissue factor is exposed to blood by vascular injury or plaque rupture, it binds to factor VII/VIIa in the presence of Ca^{++} . The

complex of tissue factor and factor VII/VIIa activates factor X to factor Xa, which in turn, activates prothrombin to thrombin in the presence of factor Va, phospholipid and Ca^{++} . The resulting thrombin converts soluble fibrinogen to insoluble fibrin which deposits as clots.

5 The complex of tissue factor and factor VII/VIIa not only activates factor X but also activates factor IX to form factor IXa. Factor IXa activates factor X in the presence of phospholipid, factor VIIIa and Ca^{++} . The reactions, except for the conversion of fibrinogen to fibrin, require negatively charged phospholipid, such as phosphatidylserine (PS), for optimal catalysis and proceed at the surface of insoluble phospholipid to 10 localize fibrin clot formation. Thus, major components of fibrin clots are insoluble fibrin, phospholipid and the activated coagulation factors.

15 Phosphatidylserine, which is highly thrombogenic *in vitro*, generally is absent from the external face of the plasma membrane in both erythrocytes and platelets. The asymmetry is maintained by an active transport mechanism. It is believed that the asymmetrical distribution of PS 20 is altered by platelet activation and PS becomes exposed on the external face of the plasma membrane. That reorientation provides negatively 25 charged phospholipids for the formation of the

prothrombinase complex and also enhances other phospholipid-dependent reactions in the coagulation cascade.

5 The coagulation factors that contain gamma-carboxyglutamic acid residues (factors X, IX, VII and prothrombin) bind to negatively charged phospholipids with binding constants in the 10^{-6} - 10^{-7} M range in the presence of Ca^{++} . The major source of negatively charged phospholipids for 10 blood coagulation *in vivo* is thought to be the platelet plasma membrane. In fact, factor Xa binds to the surface of activated platelets where it forms a complex (prothrombinase) with platelet-bound factor Va to activate prothrombin.

15 Plasmin, a serine protease, is the sole plasma enzyme responsible for fibrin dissolution. It circulates in blood as a precursor, plasminogen. Plasminogen is a single-chain polypeptide that is converted to the two-chain active form, plasmin, by 20 plasminogen activators. Plasmin is composed of an NH_2 -terminal A-chain and a COOH -terminal B-chain held together by a disulfide bond. The A-chain contains five characteristic repeating units (kringle domains) while the B-chain contains the serine-protease catalytic unit. The region from 25 the first kringle through fourth kringle binds to fibrinogen and some of the circulating plasminogen coprecipitates with fibrin when a clot is formed.

Plasminogen that is proteolytically cleaved (Lys-plasminogen) has a higher affinity for fibrin clots than intact plasminogen (Glu-plasminogen) and thus accelerates lysis of fibrin clots. The fibrin binding sites of plasminogen are located in the 5 first and fourth kringle domains.

Tissue-type plasminogen activator (tPA) and urokinase (uPA) are two physiologic activators of plasminogen. Both activators are synthesized as 10 single-chain zymogens and are converted into two-chain active forms. tPA is a membrane bound protein synthesized mainly in endothelial cells and released into the blood stream in response to certain stimuli. The secretion of tPA into the 15 blood stream triggers extrinsic fibrinolysis. The NH₂-terminal chain of tPA contains a finger domain, a growth factor-like domain and two kringle domains. The second kringle domain has binding affinity (K_d , also known as binding constant or 20 affinity constant) for the fibrin clot of 1.6×10^{-7} M. The catalytic efficiency of tPA is about 1,000 times higher toward fibrin-bound plasminogen than circulating plasminogen.

Prourokinase (single-chain urokinase or 25 scuPA), a precursor of urokinase, is present in blood at low concentrations. Prourokinase is activated by plasma kallikrein and plasmin to stimulate intrinsic fibrinolysis. Although scuPA

has a kringle domain, it shows little binding affinity for fibrin clots. However, it hydrolyzes fibrin-bound plasminogen more efficiently than free plasminogen.

5 A bacterial protein, streptokinase (SK), forms a stoichiometric complex with plasminogen which converts plasminogen to plasmin.

10 Fibrinolytic agents, tPA, uPA, scuPA and SK are being used as therapeutic agents to treat patients suffering from thrombosis. Although the agents represent a major advance, problems remain due to short half-life in circulation and a propensity to cause systemic fibrinogenolysis. For example, the relatively insufficient binding affinity for fibrin and probable cross-binding to circulating fibrinogen force a high-dose administration of tPA, which causes a significant degree of fibrinogenolysis.

15 The above therapeutic proteins have been modified in attempts to overcome the above-noted problems. The strategies used for improvements include: making the molecules resistant to circulating inhibitors; strengthening binding affinity for fibrin clots; and targeting fibrin deposits by conjugating plasminogen activator with antibody specific to fibrin clots.

20 Acylated plasminogen/SK (APS) has a higher fibrin selectivity than SK. The APS conjugate is

not inactivated by a α_2 -plasmin inhibitor because the hydroxy group of the active site serine residue is blocked. APS binds to fibrin clots and its acyl group is cleaved slowly to produce the active form. The half-life of APS is significantly longer than that of the unmodified parent molecule.

5 A truncated scuPA (residues 1-143 deleted) which lacks the growth factor and kringle domains in the heavy chain was expressed. The molecule has a fibrin selectivity identical to the intact form but is not inhibited by plasminogen activator 10 inhibitor-1 (PAI-1).

15 A modified tPA where the binding site (residues 296-302) to PAI-1 was deleted by site-directed mutagenesis was also expressed. The molecule has the same enzyme activity as the native tPA but had a strong resistance to inhibition by PAI-1 and other serpins in circulating blood.

20 A chimeric molecule that combined the fibrin binding domain of plasminogen (A-chain) with the catalytic domain of urokinase had a eight-fold higher binding affinity for fibrin clots than urokinase. It also had a higher catalytic activity toward the fibrin monomer.

25 A chimeric molecule composed of the A chain of plasminogen and the catalytic domain of tPA had the same binding affinity for fibrin as plasminogen and the same catalytic activity as native tPA.

Murine monoclonal antibodies specific to the beta-chain of fibrin were conjugated with the catalytic chain of tPA or scuPA using a disulfide cross-linking agent. The antibodies have dissociation constants on the order of 2×10^{-7} M. The antibody/tPA conjugate was ten times more active than native tPA in lysis of the fibrin monomer. An antibody/urokinase conjugate showed 1,000 times higher activity than urokinase.

Several proteins with anticoagulatory activity have been isolated from human placenta. The proteins were found to be members of the lipocortin/annexin family and to date, eight members of the family have been isolated from various tissues and cultured cells with many different functions proposed. The proteins are given the common name, "annexin". All annexins share the property of calcium-dependent binding to anionic phospholipids. Funakoshi et al., Biochem. (1987a) 26, 5572-5578; Tait et al., Biochem. (1988) 27, 6268-6276; Römisch and Heimburger, Biol. Chem. Hoppe-Seyler (1990) 371, 383-388.

Annexin V (also known as PAP-I) is a major component of the family and is isolated from placenta. It contains one free sulphhydryl group and does not have any attached carbohydrate chains. The primary structure of annexin V deduced from the cDNA sequence shows that annexin V comprises four

internal repeating units (each unit has 60-80 amino acid residues). EPA 0 279 459; U.S. Pat. No. 4,937,324; Funakoshi et al., Biochem. (1987b) 26, 8087-8092. Among annexins, annexin V has the 5 strongest binding affinity ($K_d < 10^{-10}$ M) for phospholipid vesicles containing 80% phosphatidylcholine (PC) and 20% PS under conditions that are comparable to plasma and extracellular fluid (1.2 mM ionized calcium, 0.15 M 10 ionic strength). Annexin shows high affinity for membranes containing PS and phosphatidic acid (PA), phospholipids carrying two negative charges. Tait & Gibson (1990) Cytokines Lipocortin Inflam. Diff., pp. 173-181. Binding is reversible and completely 15 calcium-dependent.

Annexin V binds to human platelets. Unstimulated platelets express a small number of binding sites, but the number of binding sites is increased greatly by certain platelet agonists (for example, approximately 15-20 fold by a combination 20 of thrombin and collagen). There are approximately 100,000 binding sites per platelet after stimulation with thrombin and collagen. The binding sites have an apparent dissociation 25 constant (K_d) of 7 nM. The binding is calcium-dependent, reversible and can be inhibited completely by PS-containing vesicles. Annexin V also can displace previously bound factor Xa from

the platelet surface. Thiagarajan & Tait, J. Biol. Chem. (1990) 265, 17420-17423.

Annexin V inhibits all of the activation reactions in the coagulation cascade where phospholipid is involved. -- To catalyze the reactions, gamma-carboxyglutamic acid-containing coagulation factors bind to negatively charged phospholipids in the presence of Ca^{++} . The dissociation constants of the γ -carboxyglutamic acid-containing coagulation factors for phospholipid are in the 10^{-6} - 10^{-7} M range, which is three to four orders of magnitude weaker than that of annexin V. (For the purposes of the instant invention, greater dissociation constants are those with greater numerical molarity values, thus a constant of 10^{-8} M is greater than a dissociation constant of 10^{-10} M. But of course, for the present invention, a dissociation constant of 10^{-10} M represents greater binding propensity than a constant of 10^{-8} M). The inhibition mechanism of annexin V is to compete with the coagulation factors for binding to anionic phospholipids.

Levels of annexin V in human plasma and cells in contact with blood were measured by ELISA using an affinity purified rabbit antiserum. Annexin V is present intracellularly in platelets, endothelial cells and leukocytes but is absent in erythrocytes. Annexin V essentially is not present

10

in normal human plasma and can be released by cell damage or death. Thus the protein that appears to be intracellular under most normal conditions can be released into the extracellular milieu with cell damage or death. Flaherty et al., J. Lab. Clin. Med. (1990) 115, 174-181.

5 In order to further improve fibrinolytic agents, a higher binding affinity for thrombi is desirable. The affinity of annexin V for negatively charged phospholipids is approximately 10 50 times stronger than that of tPA for fibrin and 10-100 times stronger than that of fibrin-specific antibodies.

SUMMARY OF THE INVENTION

15 An object of the invention is to provide annexin-plasminogen activator conjugates useful for thrombolysis.

20 Another object of the instant invention is to provide methods for making annexin-plasminogen activator conjugates.

25 Another object of the instant invention is to provide therapeutic compositions and therapeutic methods comprising annexin-plasminogen activator conjugates for treating disorders resulting from thrombosis.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a map of recombinant plasmid pMT017. The trp promoter region is denoted as trp p/o, the prourokinase coding region as ProUK, the hirudin linker as HIRUDIN, the annexin V coding region as AnV and the trp attenuator as trpA. Commonly known and useful restriction sites are denoted.

Figure 2 depicts a map of recombinant plasmid pMT024. The Pichia AOX promoter region is denoted as 5'Aox1, the scuPA-hirudin-annexin V coding region is denoted as PPA/HIRUDIN/AV, the AOX terminator region is denoted as 3'Aox1 and the yeast selectable marker is denoted as HIS4. The solid black box just downstream from the XhoI site of the 5'Aox1 sequence represents the Mucor rennin signal sequence. The 2.86 kb sequence between the 3'Aox1 and 5'Aox1 sequences are pBR322-derived sequences. Sizes are noted in kilobases (kb) and commonly known and useful restriction sites are denoted.

Figure 3 depicts a map of recombinant plasmid pMR391. Beginning with the HindIII site, the first narrow right slanting cross hatched region is the SV40 enhancer and promoter, the second narrow left slanting cross hatched region is the SV40 splice junction, the third clear region is the PPA (scuPA)

coding region, the fourth wide right slanting cross hatched region is the hirudin linker, the fifth wide left slanting cross hatched region is the annexin V coding region and the sixth region downstream from the XhoI site is the SV40 polyA region.

DETAILED DESCRIPTION OF THE INVENTION

The annexins may be isolated from a variety of tissue extracts. Funakoshi et al. (1987a) supra; (1987b) supra; Tait et al. (1988) supra; U.S. Pat. No. 4,937,324. Suitable tissues include liver, lung and placenta. A particularly suitable tissue is human placenta. Briefly, the tissue is cut into small pieces and washed with chilled physiologic buffer, such as phosphate buffered saline or 50 mM Tris-HCl, pH 7.9 containing 50 mM NaCl. The tissue chunks are homogenized in a blender generally in a physiologic buffer containing 5 mM EDTA and 5 mM benzamidine. The homogenate is filtered to obtain a filtrate.

The filtrate is next exposed to a precipitation procedure, for example ammonium sulfate. Ammonium sulfate is added to the filtrate to a saturation from about 30% to about 50%. Precipitates formed therefrom are removed by centrifugation. Additional ammonium sulfate is

then added to the supernatant to a concentration for about 70% to about 90% and any precipitates formed therefrom are collected by centrifugation. The precipitates are pooled and dissolved in a physiologic buffer, such as those noted above, and the resulting solution is dialyzed overnight against large volumes of physiologic buffer to remove the ammonium sulfate. The buffer is changed at regular intervals.

The dialysate then is passed over an anion exchange medium such as DEAE coupled to, for example, Sephadex, cellulose and Sepharose. Adsorbed proteins are eluted with a linear gradient of increasing salt concentration, for example from about 50 mM to about 500 mM NaCl. Annexin-containing fractions (assay described below) are pooled.

Pooled fractions are concentrated for example, by repeat ammonium sulfate precipitation, polyethylene glycol precipitation or ultrafiltration. If necessary the samples are dialyzed against the physiologic buffer. The samples are then passed over a gel filtration column. A suitable gel filtration column comprises a matrix of Sephadex G-75. Active fractions were collected, pooled and dialyzed to reduce the salt concentration.

The dialysate then is exposed to a cation exchange medium. Suitable cation exchange media include CM-Sephadex, SP-Sephadex, CM-cellulose or Mono S (Pharmacia). The adsorbed proteins are eluted with a buffer gradient of increasing salt concentration. Active fractions are pooled and concentrated as described above.

If placenta is used as a tissue source, several different species of annexin can be obtained. In the above described purification scheme, the species are distinguishable following the ion exchange chromatography and thus the species are distinguishable by elution at differing salt concentration.

Degree of purification can be determined by, for example, SDS-PAGE under reducing or non-reducing conditions using procedures that are recognized in the art. The annexins obtained from placenta so far identified have molecular weights in the range of 30,000 to 35,000. Accordingly, gels of appropriate polyacrylamide concentration are selected.

Several different characteristics of annexins can be monitored. For example, annexin shows an anti-coagulant activity. One such anti-coagulant assay method incorporates rabbit brain cephalin. One vial of cephalin (Sigma) is suspended uniformly in 100 ml of saline. Equal volumes of cephalin and

0.033 M CaCl_2 are mixed. In another tube acid washed kaolin is suspended in saline at a concentration of 5 mg/ml.

5 Then 20 μl of pooled normal human plasma, 20 μl of the kaolin suspension and 10 μl of test sample were mixed and incubated for 10 minutes at 37°C. Finally, 40 μl of the cephalin-calcium mixture is added and clotting time is determined.

10 Assays that measure inhibition of the intrinsic coagulation (kaolin-induced coagulation) pathway can be modified readily to measure inhibition of the extrinsic coagulation pathway (Kondo et al., Thromb. Res. (1987) 48, 449-459. Clotting time is determined, for example by the 15 method described above except that the kaolin suspension is replaced by thromboplastin. Human brain thromboplastin is diluted with 50 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl to obtain a controlled clotting time of approximately 20 60 seconds.

Annexins also show an affinity for phospholipids. One method for quantifying the degree of annexin binding to phospholipids is founded on fluorescence quenching. (Tait et al., 25 J. Biol. Chem. (1989) 264, 7944-7949) Annexin (50 μM) is labeled by incubating with fluorescein isothiocyanate (50 μM) for one hour at 37°C in a buffer of 0.05 M borate, pH 9.0, 0.15 M NaCl, 1 mM

EDTA. The reaction mixture is dialyzed against 20 mM HEPES pH 8.0 buffer. The dialysate is applied to a cation exchange column and eluted with a linear salt gradient. Annexin labeled with one fluorescein molecule eluted at 0.27 M NaCl and multiple fluoresceinated forms of annexin elute at salt concentration between 0.30 M and 0.45 M NaCl.

5 Next, phospholipid vesicles are prepared by adding approximately, 20% PS, 20% diheptanoyl-phosphatidylcholine and 60% long chain 10 PC resulting in the spontaneous formation of unilamellar vesicles. Aliquots of phospholipid stock solutions in chloroform are mixed to yield the desired molar ratios and the chloroform is removed by evaporation under nitrogen. The 15 phospholipids are then dissolved in HEPES buffer by sonication for 3 minutes on ice followed by overnight equilibration at 4°C.

20 Fluorescence measurements can be performed in a fluorometer (for example, SLM 8000 C/Aminco, Urbana, Illinois). The appropriate wavelengths are selected, for example, for fluorescein the excitation wavelength is 495 ± 16 nM and emissions are monitored at 520 ± 10 nM.

25 Binding assays are performed in standard quartz fluorescence cells containing a buffer and the fluorescence labeled annexin. The solution is mixed once by inversion and then varying amounts of

phospholipids are added to the cuvette. The contents are mixed again by inversion and fluorescence intensity is recorded. Following addition of 5 mM EDTA to the cuvette, fluorescence intensity is recorded again. The degree of quenching is calculated from the ratio of the final fluorescence intensity to fluorescence intensity in the presence of EDTA.

Binding of annexin to phospholipid vesicles occurs with high affinity ($K_d < 10^{-10}$ M) under conditions that are comparable to that of plasma and extracellular fluid (1.2 mM ionized calcium, 0.15 M ionic strength). Binding is reversible and completely calcium dependent.

Other assays that are suitable for monitoring the presence of annexin include for example antibody-based methods such as ELISA and Western blot.

Alternatively, annexin can be produced by recombinant methods. cDNA clones can be obtained using antibody screening of expression libraries or using oligonucleotide probes deduced from annexin peptides. A full length cDNA clone of annexin V has been obtained and subcloned in expression vectors. Funakoshi et al. (Biochem. (1987a) 26, 5572-5578; (1987b) 8087-8092) used an affinity-purified antibody to screen a cDNA bank to obtain an annexin V clone. A 1.3 kb NcoI/HindIII

fragment containing the coding sequence of annexin V was cloned into the expression vector pKK233.2 (Pharmacia) to form the expression plasmid pPAP-I-wt. The recombinant annexin is expressed 5 cytoplasmically at a level of about 2% of cellular protein. The recombinant annexin is obtained from host cells using procedures recognized in the art.

Annexin V contains four tandem, imperfect repeats of about 75 amino acid residues (Funakoshi et al. Biochem. (1987b) 26, 8087-8092). The 10 repeats contain conserved amino acid residues at 4 sites, 15 positions comprise hydrophobic amino acid residues, 4 positions comprise hydroxyamino acids and 2 sites comprise acidic amino acids.

15 Each of the four repeating sequences contains two regions that are commonly present in phospholipid binding proteins. The first region, the NH₂-terminal 17 residues, conforms to a consensus sequence (Geisow et al., Nature (1986) 320, 636-638) of Lys-Gly-X-Gly-Thr-Asp-Glu-X-X-h-h-X-h-h-X-Ser-Arg (SEQ ID NO:1), where h represents hydrophobic amino acids and X can be any amino acid. The sequence has been found in Ca⁺⁺ regulated 20 membrane binding proteins such as endonexin and calelectrin. Phospholipase A₂ and a viper venom phospholipase A₂ inhibitor also have closely related 25 sequences. The second homologous region in the proteins is a stretch of six residues of

hydrophobic amino acids at the C-terminal portion of each repeat. The two regions are thought to be involved directly in binding to phospholipid (Geisow, FEBS Lett. (1986) 203, 99-103). The 5 strong anticoagulant activity can be attributed to the presence of the phospholipid binding regions. The crystallographic analysis of annexin V showed that the four domains of annexin V have a similar folded structure, each consisting of five α helices 10 (Huber et al., EMBO J. (1990) 9, 3867-3874). The same group also found three strong calcium binding sites of annexin V located on the convex face of the I, II and IV repeats. Huber et al. suggest the calcium binding sites mediate the phospholipid 15 binding to the annexin V molecules (Huber et al. FEBS Lett. (1990) 275, 15-21).

The annexin molecule can be subdivided or altered at one or more amino acid residues so long as the phospholipid binding capability is not 20 reduced substantially. Thus annexin can be truncated, for example, to include one or more domains or contain fewer amino acid residues than the native protein, or can contain substituted amino acids. Any changes are acceptable within the 25 scope of the invention so long as the mutein or second generation annexin molecule does not contain substantially lower affinity for phospholipid.

Substantially lower affinity is a binding constant for phospholipid that is greater than about 10^{-7} M.

Similarly, the fibrinolytic agent can be modified or altered within the scope of the invention so long as the resulting agent retains the capability of lysing fibrin clots. For example, point mutated, point deleted, point substituted and/or truncated fibrinolytic agents, such as tPA, urokinase or prourokinase in which one or more domain(s) has (have) been deleted, can be employed herein. See Wikstrom et al., *Fibrinolysis* (1990) 5, 31-41 and Van Zonneveld et al., *Proc. Natl. Acad. Sci. USA* (1986) 83, 4670-4674 on deletion tPA molecules. Other tPA deletion products as well as urokinase and prourokinase deletion molecules are contemplated for use herein. EP 266032A, EP 299706A, EP 308716A, EP 236040A, EP 247674A, EP 253241A, US 4,753,879, WO 87/04722 and JPA 63/230084, among others, describe various altered or modified prourokinase, urokinase and tPA molecules. US 4,752,581, US 4,908,204, US 4,992,274, US 4,916,071 and EP 2311883A describe hybrid molecules such as urokinase/tPA molecules.

The proteolytic properties of modified fibrinolytic agents can be assessed in known assays such as using the chromogenic substrates, S-2288, S-2444 or S-2251 (Helena Laboratories) as taught in Schnee et al. (*Proc. Natl. Acad. Sci. (1987)* 84,

6904-6908) or as taught in Bode et al. (J. Biol. Chem. (1987) 262, 10819-10823). A reduction of fibrinolysis activity of up to about 50% in a modified fibrinolytic agent relative to the native protein is contemplated to fall within the scope of the instant invention.

5 Among the many properties of annexin and particularly annexin V that is beneficial to the instant invention is affinity for thrombus or thrombi. Annexin V can target a thrombus in vivo. Radiolabeled annexin V was administered intravenously and the localization of radio activity was determined. A substantial accumulation was noted at the thrombus with a 10 thrombus:blood ratio of about 17:1 at 100 minutes 15 after-injection. The radiolabeled annexin V was rapidly cleared from the blood.

20 Thus annexin can be used to target a thrombus wherein it may exert its anticoagulant activities or annexin can serve as a means for targeting a 25 second molecule conjugated thereto. Suitable second molecules include thrombolytic agents such as tissue plasminogen activator, streptokinase, urokinase and prourokinase. Annexin conjugates are particularly useful for thrombolytic agents that are not fibrinophilic such as urokinase. Many thrombolytic agents, such as streptokinase, urokinase, prourokinase and tissue plasminogen

activator, are available commercially, either produced recombinantly or purified from natural sources. (For example, see U.S. Pat. Nos. 4,853,330; 4,766,075 for tPA; Williams, Brit. J. Exp. Path. (1951) 32, 530 and U.S. Pat. Nos. 2,989,440, 2,983,647 and 3,081,236 for urokinase; Christensen, Gen. Physiol. (1954) 28, 363 and U.S. Pat. Nos. 3,138,542, 3,226,304, 3,016,337 and 3,107,203 for streptokinase; and for prourokinase see EPA 0 139 447.)

Through selective proteolytic digestion or subcloning, second generation forms of thrombolytic agents have been produced with the advantages of having enhanced fibrin binding and enhanced half-life, for example, while minimizing adverse side effects. Thus, tPA lacking the epidermal growth factor and finger domains or containing just the second kringle and serine protease domains have been made. Also, chimeric thrombolytic agents, for example comprising portions of urokinase and tPA, are active in activating plasminogen (Pierard et al., J. Biol. Chem. (1987) 262, 11771).

Annexins can be cross-linked chemically with thrombolytic agents. For example, U.S. Pat. No. 4,564,596 teaches the use of an aliphatic diamine to conjugate urokinase with fibrinogen. U.S. Pat. No. 4,536,391 teaches a plasmin urokinase complex using a coupling reagent of the succinimide ester

variety. Sevilla et al. (Biochem. Biophys. Res. Comm. (1985) 130, 91-96) teaches the conjugation of urokinase and an anti-human fibrinogen antibody, bridging the two elements with the heterobifunctional coupling reagent, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). There urokinase was reacted with SPDP and separately the antibody was mixed with 2-iminothiolane. The SPDP-modified urokinase was then mixed with the iminothiolated antibody to produce the conjugates. The conjugates were purified by affinity chromatography.

Hashida et al. (J. Appl. Biochem. (1984) 6, 56-63) teach a number of maleimide compounds useful for conjugating proteins. Briefly scuPA, SK or plasminogen is incubated with sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) in a phosphate buffer, pH 7.0. SMCC binds covalently to free amino groups preferably to the NH₂-terminal amino group under these conditions. The reaction mixture then is applied to a gel filtration column to remove side products.

The number of SMCC molecules that are bound to the protein of interest is assessed using 4,4'-dithiodipyridine. When 1.5 molar excess of SMCC is used, the incorporation of 1.2-1.5 moles of SMCC into protein is expected according to established methods. Next, SMCC-protein, for

example SMCC-scuPA, SMCC-SK or SMCC-plasminogen, is incubated with annexin V in a phosphate buffer, pH 6.0, containing 6 M urea or 6 M guanidine. The SMCC-proteins bind specifically to the single free sulfhydryl group present at the C-terminal end (fifth residue from the C-terminus) of the annexin V molecule. The reaction product is applied to a gel filtration column (for example, Sephadex G-100) to isolate the monomeric form, which is expected to be the major product under those conditions. (The annexin V/plasminogen hybrid is activated to form annexin V/plasmin by urokinase. Annexin V is resistant to mild digestion by most proteases under native conditions.)

The compounds for conjugating annexin and thrombolytic agent can be varied to provide a spacer should steric effects compromise either annexin binding affinity or thrombolytic agent activity or both. For example Hashida et al. (supra) teaches compounds with variable numbers of methylene group between the maleimide and succinimide functions and the diamine bridging agent of Maximenko et al. (supra) comprises an aliphatic diamine with 1-12 methylene groups.

Similarly, spacer regions can be configured into recombinant conjugates by inserting

appropriate coding sequences between the annexin and thrombolytic agent coding sequences.

Conjugates can also be prepared recombinantly as many of the above recited thrombolytic agents have been cloned. For example, Schnee et al. (Proc. Natl. Acad. Sci. (1987) 84, 6904-6908) teach the expression of a fibrin monoclonal antibody-tPA conjugate by recombinant means. Thus, the heavy chain gene of the anti-fibrin antibody was inserted adjacent to the tPA β -chain gene in an expression vector and recombinant hybrid protein was produced. Pierard et al. (J. Biol. Chem. (1987) 262, 11771-11778) constructed a series of recombinant plasminogen activators comprising domains obtained from urokinase or tPA. The corresponding nucleic acid sequences to the different domains were excised and recombined to produce chimeric coding sequences that in turn produced chimeric protein.

The artisan determines the host in which to express recombinant conjugates. For example, E. coli, B. subtilis, yeast and mammalian cells can be used. The cassette containing the coding sequence is placed into the operable site of an appropriate expression vector comprising the necessary host cell recognizable 5' non-coding sequences including promoters and downstream 3' non-coding sequences. A suitable E. coli expression vector is pKK233-2 (Amann & Brosius, Gene (1985) 40, 183-190); a

suitable yeast expression vector is DPOT (Thim et al., Proc. Natl. Acad. Sci. 83, 6766-6770); and a suitable mammalian cell expression vector is pDSP1.1BGH (Pfarr et al., DNA (1985) 4, 461-467).

5 The domains discussed above may contribute jointly or severally to phospholipid binding and the anticoagulant activity. Accordingly, nucleotide coding sequences of one or more domains or duplicates of one domain can be subcloned adjacent to the coding sequence of the thrombolytic 10 agent to produce conjugates with novel or enhanced properties.

15 The conjugates can comprise the active component of pharmaceutic compositions. Such compositions would contain pharmaceutically acceptable carriers, diluents and excipients. For example, suitable carriers include buffers, physiologic saline, tissue culture medium and water. The conjugates can be administered using 20 art recognized methods such as intravenous infusion. The treatment regimen is determined empirically from animal studies and clinical trials, and is keyed to the severity of the disease, physical condition of the patient and the like. The artisan can obtain suitable guidance 25 from many of the treatises in pharmacology such as, "Goodman & Gilman's The Pharmaceutical Bases of

"Therapeutics" (6th Ed., Goodman et al., eds., MacMillan Publishing Co., New York 1980).

5 In another embodiment, the targeting capability of annexin can be used to deliver pharmaceutics to local sites. Thus the artisan can use the methods described herein to conjugate a pharmaceutic to annexin. For example, a cytotoxic agent, mitogen, antibiotic and the like can be used. As with other pharmaceutics, delivery route and dosage can be ascertained by the artisan 10 practicing methods known in the art.

The invention will be exemplified further in the following non-limiting examples. Amounts are stated in terms of w/w or w/v.

15

EXAMPLE I

Iodinated annexin V is prepared using standard procedures such as methods using the Iodogen reagent. Thiagarajan and Tait, J. Biol. Chem. (1990) 265, 17420-17423. The animal model of 20 arterial thrombosis is essentially that described earlier for dogs but adapted to rabbits (Ritchie et al., Circulation (1986) 73, 1006-1012) as described above. A rabbit is intubated, anesthetized with 1-2% halothane and monitored continuously by 25 electrocardiogram. The artery of interest (carotid or femoral) is exposed and a 2-5 cm section is

isolated between two ligatures. The section is crushed repeatedly with forceps, the proximal ligature is released for 1 minute to allow fresh blood to enter and then retightened. After two hours, radiolabeled I¹²⁵-annexin V is injected as a bolus via a peripheral vein and the ligatures released thirty seconds later. After a variable period to allow accumulation of the annexin V at the clot and clearance of unbound annexin V from the blood pool, the artery is removed, fixed in formalin, sectioned and measured for radioactivity.

5

10

EXAMPLE II

PCR (U.S. Pat. Nos. 4,683,195; 4,683,202) is used to amplify an annexin V cDNA with a 5' NcoI terminus and a 3' XbaI terminus obtained from the plasmid pPAP-I-1.6. Likewise, a scuPA cDNA is amplified by PCR with a 5' XbaI site and a 3' HindIII site. Primers for scuPA are designed from the published cDNA (Holmes et al., Bio/Tech (1985) 3, 923-929) and amplification can be from a cDNA library. The two PCR products are digested with NcoI and XbaI; and XbaI and HindIII, respectively, and are then ligated into an E. coli expression vector such as pKK233-2 (Amann & Brosius, Gene (1985) 40, 183-190) previously digested with NcoI and HindIII. The plasmid is placed into an

15

20

25

appropriate host such as DH5 α . Recombinants are identified by colony hybridization, and the construction verified by restriction mapping and DNA sequencing. The expression level of the hybrid 5 protein in *E. coli* is then optimized by varying incubation temperature and the length of the induction period with IPTG.

It is likely the hybrid protein will be packaged in inclusion bodies as occurs with 10 annexin V and scuPA individually (Winkler & Blaber, Biochem. (1986) 25, 4041-4045). The inclusion bodies are isolated by centrifugation (10 minutes at 10,000 x g), solubilized in 6 M urea and renatured by dialysis. The hybrid protein then is 15 purified by conventional low-pressure and FPLC chromatographic methods, with fractions monitored by fluorescence polarization immunoassay using anti-annexin antibody. The hybrid protein can be characterized in the same manner as described for 20 chemically produced hybrids.

An alternative approach of expression is through a secretory pathway in yeast with, for example, the vector DPOT (Thim et al., Proc. Natl. Acad. Sci. (1986) 83, 6766-6770). Both annexin V 25 and scuPA (Zaworski et al., Gene (1989) 85, 545-551) have been expressed successfully in *Saccharomyces cerevisiae*.

EXAMPLE III

Cultures of *E. coli* containing the desired plasmid are grown overnight at 37°C in L broth containing 100 µg/ml ampicillin. Cultures are 5 diluted 1:10 in 1 liter of the same medium and grown at 37°C with shaking until the A_{600} is > 0.3. Synthesis then is induced by adding IPTG to 3 mM and growth continued for 4 hours. Bacteria are harvested by centrifugation, washed once with 10 100 ml PBS containing 10 mM EDTA and stored at -20°C. Pellets are lysed by sonication for 2-3 minutes on ice in 25 ml of a buffer consisting of PBS, 10 mM EDTA, 6 M urea, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 1% Triton X-100 and 0.2 mM 15 phenylmethanesulfonyl fluoride. The extract is centrifuged (20 minutes at 25000 x g). The supernatant is dialyzed at 4°C against a buffer comprising 50 mM Tris-HCl, pH 8.0, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin, 1 mM EDTA, 3 mM NaN₃, and 100 mM NaCl, with repeated buffer changes. The 20 dialysate is membrane filtered and is applied to an affinity column, for example, an anti-annexin column, at 4°C. The column is washed with at least 100 ml of the same buffer used for dialysis and bound proteins are eluted with 0.1 M glycine, 25 pH 2.5 buffer. The eluate is collected in one-fourth volume of 1 M Tris-HCl, pH 8.0 for

immediate neutralization of pH and then subjected to dialysis and concentration.

EXAMPLE IV

5 Human annexin binds to rabbit platelets in a manner similar to when annexin binds to human platelets, indicating that the rabbit can be used as an experimental model to test the activity of annexin V hybrids.

10 Thrombi were induced experimentally by mechanical trauma and stasis in the carotid artery of a rabbit. Radiolabeled annexin V (¹²⁵I-annexin V, 150 uCi) was given as a single 15 intravenous injection 2 hours later. Serial blood samples were then taken at 5, 10, 15, 30 and 60 minutes to determine blood clearance rate; the animal then was sacrificed at 100 minutes and samples of blood, urine and tissue were taken for measurement of weight and ¹²⁵I content. No signs of acute systemic toxicity were noted.

20 As can be seen in Table I, the ¹²⁵I-annexin V accumulated substantially at the thrombus, with a thrombus:blood ratio of 17:1 at 100 minutes after injection. Accumulation in intact blood vessels (aorta and vena cava) and most other tissues was 25 minimal. The ¹²⁵I-annexin V was cleared rapidly from the blood with a half-life of 10 minutes.

Clearance was primarily through the kidney as indicated by the accumulation of radioactivity in the urine and kidney.

TABLE I

5 Biodistribution of ¹²⁵I-Annexin V in a Rabbit with Arterial Thrombosis

	<u>Tissue or Fluid</u>	<u>Counts per sec per gm specimen</u>	<u>Ratio specimen:blood</u>
	Blood	2080	1.00
10	Clot*- artery	35771	17.20
	medial portion	12980	6.24
	distal portion	5525	2.66
15	Carotid*- artery	9607	4.62
	medial portion	9833	4.73
	distal portion	4700	2.26
	Aorta	1527	0.73
	Vena cava	1620	0.78
	Kidney	60752	29.20
20	Urine	32644	15.70
	Liver	1506	0.72
	Spleen	3141	1.51
	Heart	1905	0.92
	Lung	4893	2.35
25	Skeletal muscle	1524	0.73

* The thrombosed carotid artery was removed and fixed in formalin. It was then sectioned in three pieces; for each piece, the thrombus was removed and the vessel and thrombus counted separately.

EXAMPLE V

Essentially identical results, as that presented in Example I, were obtained when thrombosis was induced in the femoral rather than 5 the carotid artery.

EXAMPLE VI

Annexin/scuPA and annexin/plasminogen are activated by plasma kallikrein (or plasmin) and 10 urokinase, respectively. Activated annexin/urokinase, annexin/plasmin and annexin/SK are incubated with plasminogen and the resulting 15 plasmin is assayed using the synthetic substrate, Boc-Glu-Lys-Lys-MCA (Peptides International). The plasminogen activating activities of the hybrid molecules can be compared with that of the parent 20 proteins.

The fibrinolytic activity of hybrids can be assayed by the method of Rijnen et al. (Thromb. 25 Haemostas. (1984) 52, 308-310), except that platelet-rich plasma is used instead of platelet-poor plasma. Fresh human platelet-rich plasma is added to thrombin (or tissue factor), CaCl_2 and ^{125}I -fibrinogen, immediately placed in silastic tubing (4 mm I.D.) and incubated at 37°C for 30 minutes. The tubing is cut into pieces of 25

defined length and clots are removed from the tube and washed with buffer. After the radioactivity is measured, the clots are incubated with the hybrids or the native proteins (for example, scuPA, SK or plasmin). Fibrinolytic activity is assessed by determining the solubilized radioactivity. The concentrations of fibrinogen and plasminogen in the sample plasma are assayed by ELISA to determine fibrinogenolysis.

10

EXAMPLE VII

15

Human single-chain urokinase-type plasminogen activator (scuPA) was obtained from the Green Cross Co., Osaka, Japan. Sulfo-SMCC [sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate] was purchased from Pierce, Rockford, Illinois.

ScuPA was dialyzed overnight against 0.1 M sodium phosphate, pH 7.3 containing 6 M urea. After dialysis, the concentration of scuPA was adjusted to 32.5 μ M.

20

25

Sulfo-SMCC was added to dialyzed scuPA aliquots at two final concentrations of 65 μ M and 325 μ M. The samples were incubated for 30 minutes at room temperature. At the end of incubation, glycine (10 mM) was added. The samples were applied to a gel filtration column (PD-10, Pharmacia) to remove excess unreacted reagents.

The column then was eluted with 0.1 M sodium phosphate buffer, pH 6.0 containing 6 M urea and 10 mM EDTA. The protein fractions were pooled (scuPA-SMCC).

5 Annexin V was dialyzed overnight at room temperature against 0.1 M sodium phosphate buffer, pH 7.3 containing 6 M guanidine (or 8 M urea). The sample was applied to a gel filtration column (PD-10) and eluted with 0.1 M phosphate buffer, pH 6.0 containing 6 M urea and 10 mM EDTA. The protein fractions were pooled (unfolded annexin V).

10

15 SMCC-scuPA was conjugated with unfolded annexin V by incubation overnight at room temperature. Aliquots of scuPA/annexin V conjugate were applied to SDS-polyacrylamide gels and were separated electrophoretically. SMCC-scuPA alone, annexin V alone and molecular weight markers served as controls. Formation of the conjugate was confirmed as a species of molecular weight equal to the sum of the molecular weights of the two components.

20

EXAMPLE VIII

25 ScuPA/annexin V conjugate as prepared in Example VII was activated by plasmin. Fifty microliters of scuPA/annexin V conjugate (approximately 500 IU/ml) were incubated with 50 μ l

of plasmin (0.9 μ M) for 10 minutes at room temperature. Then 50 μ l of aprotinin (10 μ M) were added and the mixture was incubated for 5 minutes at room temperature to inhibit plasmin.

5 Next, 50 μ l of the synthetic peptide substrate (2 mM), pyroGlu-Gly-Arg-p-nitroanilide (S-2444, Kabi, Sweden) were added and the mixture was incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 1 ml of 10% acetic acid. The amount of p-nitroaniline produced was measured by absorbance at 405 nm. The activity was expressed as International Standard Units (IU).

10 ScuPA and scuPA to which one SMCC was bound showed equivalent activity. As the number of SMCC molecules bound to scuPA increased, the activity decreased slightly. At a ratio of 10 SMCC molecules to each scuPA molecule, the conjugate had 15 a 25% reduction of activity.

EXAMPLE IX

20 scuPA cDNA can be derived from any of a number of available scuPA clones, for example, plasmid pSV-GI-preUK (JP-A-60-180591, EP-A-253241 or EP-A-154272). For joining scuPA cDNA downstream of a promoter, such as at the ClaI site of the trp 25 promoter (EP 0152830) and available in vectors pDR720 from PL Biochemicals and pGX112 from Genex,

Gaithersburg, MD, a synthetic DNA having the following base sequence (SEQ ID NO:2) can be used:

	M	S	N	E	L	H	Q	V	P	N
	XbaI									
										TthHB8I

5 5'-TCGAGCATCGATAAA ATG TCT AAC GAA TTG CAC CAA GTT CCA TCG-3'
(SEQ ID NO:2)

10 The synthetic DNA comprises the nontranslated region from the Clal site of scuPA to the ATG codon and the region ranging from the ATG codon to the TthHB8I site, which corresponds to the 10th amino acid residue, with an XbaI site upstream from the Clal site for joining to an expression vector capable of functioning in yeast as well. The synthetic DNA was ligated to the 5' terminus of
15 scuPA cDNA to enable operably linking the scuPA cDNA to a trp promoter.

20 Annexin V cDNA can be derived from, for example, the clone of Funakoshi et al., (1987b) supra, Fujikawa et al., U.S. Pat. No. 4,937,324 or plasmid pPAP-I-1.6, see Example II. The synthetic DNA having the following base sequence was used as a hirudin linker (Biol. Chem. Hoppe-Seyler (1986) 367, 731-740) for joining the C-terminus of scuPA to the N-terminus of annexin V. The hirudin linker
25 (synthesized using known techniques) comprises a base sequence encoding the 50th to 64th amino acid

residues of the amino acid sequence of hirudin
(SEQ ID NO:3).

uk←| |→hirudin

5 Arg Ile Arg Ser His Thr Lys Glu Glu Asn Gly Leu Ala Leu Ser His Asn Asp Gly Asp
AGG ATC CGC AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC TCT CAC AAC GAC GGC GAC
BamHI

hirudin←| |→annexin V

10 Phe Glu Glu Ile Pro Glu Glu Tyr Leu Ala Gln Val Leu Arg
TTC GAA GAA ATC CCG GAA GAA TAC CTG GCA CAG GTT CTC AGA
Nsp(7524)V DdeI

(SEQ ID NO:3)

15 A suitable strain of *E. coli* and a vector
operable therein are selected. For example,
plasmid pMT017 (Figure 1) was constructed using
plasmid pYN5, which was prepared by inserting a trp
promoter/operator (p/o) into pBR322 (JP-A-60-160887
or EP-A-158230); and a second plasmid comprising a
20 sequence prepared by ligating BglII linkers at both
ends of trpA (trp attenuator which is a terminator
having a polyT tail, commercially available from PL
Biochemicals) having the following base sequence:

5'-AAAAAAAAGCCCGCTCATTAGGCGGGCT-3' (SEQ ID NO:4)

25 and inserting the resulting DNA fragment into the
BamHI site of pUC9. The DNA fragments of interest

were obtained and joined in accordance with conventional methods, for example, as described in Molecular Cloning, Cold Spring Harbor Laboratory (1982).

5 Plasmid pMT017 comprises under the control of the trp promoter, scuPA cDNA, a base sequence encoding 15 amino acid residues corresponding to the 50th to 64th amino acid residues of hirudin, annexin V cDNA and the trp attenuator.

10 Plasmid pMT017 was introduced into an appropriate E. coli host, such as E. coli HB101 (Takara Shuzo, following the manufacturer's recommended procedures), and the resulting transformants were incubated overnight at 30°C in
15 L broth medium supplemented with 20 µg/ml ampicillin to obtain gene expression products. After the products were solubilized and reconstituted, the biological activity of the products was examined. The products were
20 prourokinase/annexin V conjugates (hybrid protein) having a molecular weight of about 85k and reactive with both anti-urokinase antibody and anti-annexin V antibody (see, for example, Flaherty et al. supra). The products possessed thrombolytic
25 activity as determined in a fibrin plate assay.

EXAMPLE X

Plasmid pMT024 (Figure 2), a *Pichia* expression vector, was constructed using plasmids pMT017 and pAO807NX, which was prepared by converting the 5 EcoRI cloning site of plasmid pAO807N (JP-A-2-104290 or EP-A-344459) to an XhoI site using conventional methods described in, for example, *Molecular Cloning, supra*. Plasmid pMT024 comprises under the control of the AOX1 promoter, 10 a base sequence corresponding to a signal sequence of *Mucor rennin* (Hiramatsu et al., *J. Biol. Chem.* (1989) 264, 16862) obtained from plasmid pJK1 (Gene (1991) 99, 235-241), scuPA cDNA, a base sequence 15 encoding 15 amino acid residues of the 50th to 64th amino acid residues of hirudin and annexin V cDNA.

Plasmid pMT024 was introduced into an appropriate *Pichia* yeast host, such as *Pichia pastoris* GTS115 (NRRL Y-15851), following NotI 20 digestion (The fragment was EtOH precipitated and suspended in TE buffer at a concentration of about 2 mg/ml. A 10 μ l portion was added to 100 μ l of competent cells. His⁺ transformants were selected.) and the transformed cells were incubated at 30°C 25 for 3 days in a medium containing 1% yeast extract, 2% Bacto Peptone and 1% MeOH to allow the cells to secrete gene expression products. The recombinant products were prourokinase/annexin V conjugates

5

(hybrid protein) having a molecular weight of about 85k and reactive with both anti-urokinase antibody and anti-annexin V antibody. The products possessed thrombolytic activity in a fibrin plate assay.

10

15

EXAMPLE XI

Plasmid pMR391 (Figure 3), a CHO cell expression vector, was constructed using plasmids pMT017 and pSV-G1-preUK, described hereinabove, in accordance with conventional methods, for example, described in Molecular Cloning, supra. Plasmid pMR391 comprises under the control of the SV40 enhancer/promoter, a SV40 splice junction, scuPA cDNA, a base sequence encoding 15 amino acid residues corresponding to the 50th to 64th amino acid residues of hirudin, annexin V cDNA and SV40 poly A signal sequences.

20

25

CHO cells were transformed with plasmid pMR391 and the transformed cells were cultivated at 37°C for 3 days in MEM supplemented with 10% FCS to allow the cells to secrete gene expression products. The products were prourokinase/annexin V conjugates (hybrid protein) having a molecular weight of about 90k and reactive with both anti-urokinase antibody and anti-annexin V

antibody. The products possessed thrombolytic activity in a fibrin plate assay.

EXAMPLE XII

SMCC-scuPA, made using the techniques described hereinabove, is conjugated with modified annexin V with an additional cysteine residue at the N-terminus by incubation overnight at room temperature. (The modified annexin V was made by ligating to the 5' end of the annexin V coding sequence an oligonucleotide encoding about ten amino acids including a cysteine residue at the amino terminal end.) Aliquots of scuPA/modified annexin V conjugate are applied to SDS-polyacrylamide gels and are separated electrophoretically. SMCC-scuPA alone, modified annexin V alone and molecular weight markers serve as controls. Formation of the conjugate is confirmed as a species of molecular weight equal to the sum of the molecular weights of the two components.

EXAMPLE XIII

Annexin V (8 nmoles) in 250 μ l of 50 mM triethanolamine (TEA)-HCl buffer, pH 8.0 containing 50 mM KCl and 1 mM MgCl₂, was mixed with 2.5 μ l of

β -mercaptoethanol (final concentration of 1%) and cooled to 0°C. Then varying concentrations of iminothiolane (10 μ l) were added to individual samples and allowed to stand for 20 min at 0°C.

5 The samples were desalted on Fast Desalting columns (Pharmacia, HR10/10) with 50 mM Tris-HCl, pH 7.4 containing 50 mM NaCl, 1 mM EDTA and 0.05% NaN₃ (the buffer was flushed with N₂ before use) and protein-containing fractions (1.1 ml) were

10 collected. The amount of newly introduced sulfhydryl group was determined using Ellman's reagent (Ellman, Arch. Biochem. Biophys. (1958) 74, 443). The clotting activity was determined as described above and the percent inhibitory activity

15 was calculated from a standard curve constructed using authentic annexin V. The moles of sulfhydryl group (SH) per mole of annexin V and the percent clotting activity are shown in Table II. The inhibitory activity of annexin V was retained after

20 modification.

Table II

Sample	SH/annexin Concentration (mole/mole)	of 2-imino- thiolane used (μ mole)	Clotting time (sec)	Activity (%)
5	1	2.31	25.0	131
	2	1.58	12.5	133
	10	0.83	5.0	134
	4	0.56	2.5	123
	5	0.37	1.25	126
	6	0.19	0.5	127
	7	0	0	132

15

EXAMPLE XIV

ScuPA (20 nmol, 200 μ l, specific activity of 417 U/mg) in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0 was mixed with varying concentrations of iodoacetic anhydride (IAA) in tetrahydrofuran at 0°C. IAA was added three times at 3 minute intervals. The sample then was applied over a Fast Desalting column HR 10/10) using 50 mM Tris-HCl, pH 7.4 containing 50 mM NaCl, 1 mM EDTA

and 0.05% NaN_3 (the buffer was flushed with N_2 before use).

The amount of iodoacetyl group (IA) introduced into scuPA was determined as follows: 5 iodoacetyl-scuPA was mixed with known amounts of reduced glutathione and the remaining glutathione was assayed using Ellman's reagent. The amidolytic activity of iodoacetyl-scuPA was assayed using the peptide substrate, S-2444, after it was activated by plasmin. Table III shows the moles of iodoacetyl group per annexin V and the amidolytic activity of iodoacetyl-scuPA. The results indicate that iodoacetyl-scuPA has the same amidolytic activity as intact scuPA.

10

15

Table III

20

Sample	Mole IA/ mole/scuPA	IAA used	Amidolytic activity (μmole)	Activity (O.D. $_{405}$) (%)
1	0.72	2.4	0.165	98
2	0.44	1.6	0.163	94
3	0.54	0.8	0.169	106
4	0	0	0.174	100

25

EXAMPLE XV

Sulphydryl-annexin V and iodoacetyl-scuPA that were prepared as described above were mixed and allowed to stand for 1 hour at 37°C in dark. The products were applied to and separated in an SDS-polyacrylamide gel and examined by immunoblot analysis using anti-scuPA and anti-annexin V antibodies (6.7 nmol of three different preparations of sulphydryl-annexin V, i.e., moles of sulphydryl group/annexin, 1.31, 0.82 and 0.57, were conjugated with 6.7 nmol of iodoacetyl-scuPA).

Bands that migrated as a species of molecular weight of about 92,000 were detected by both antibodies showing that the 92,000 species is a conjugate (hybrid protein) of annexin V/scuPA. More conjugates were formed if annexin V has a higher sulphydryl group content and no conjugate was formed when unmodified annexin V was used.

EXAMPLE XVII

Annexin V-N1 is designed to be an amino-terminally extended molecule in which the sequence of Met-Ala-Cys-Pro-Ser-Gly-Gly-Pro-Ser-Gly-Gly-Pro-Met (SEQ ID NO:5) is connected to the amino-terminal Ala residue of mature annexin V. The SH group of the third Cys residue may be used for conjugates with iodoacetyl-scuPA.

For the construction of annexin V-N1 DNA, two complementary oligonucleotides containing NcoI sites,

(5'-CATGGCATGCCGTCTGGTGGTCCGTCTGGTGGTCC-3' (SEQ ID NO:6) and 5'-CATGGGACCAACCAGACCGACCACAGACGGGCATGC-3' (SEQ ID NO:7) were synthesized, purified, phosphorylated and annealed by standard methods. The plasmid pPAP-1-319 (5 Tait & Smith, Arch. Biochem. Biophys. (1991) 288, 141) was digested with NcoI. The double-stranded oligonucleotide then was ligated into the plasmid to create plasmid, pPAP-I-N1. DNA sequencing confirmed that the recombinant plasmid contained the intended sequence. (10 The recombinant protein was expressed and purified as described (Tait & Smith, supra).

Transformed *E. coli* cells were collected from 10 liters of culture medium and washed once with 50 mM Tris-HCl buffer, pH 7.4 containing 50 mM NaCl. Cells (15) were suspended in 250 ml of phosphate buffer, pH 7.4 containing 6 M urea, 1% Triton X-100, 0.1 mM diisopropyl fluorophosphate and 10 mM EDTA and sonicated for 3 x 1 min. The cell lysate was centrifuged for 20 min at 15,000 rpm and the supernatant was collected followed by (20) dialysis against 50 mM Tris-HCl buffer, pH 7.4 containing 50 mM NaCl, 1 mM EDTA. The sample then was applied to a Mono Q column (Pharmacia) with the same buffer and eluted with an NaCl gradient (0.05-1 M). Annexin V-N1 was detected by dot-blot and the fractions containing annexin (25) V-N1 were pooled and concentrated by ultrafiltration. The concentrated sample was applied to a TSKgel G3000 SWXL (20 x 500 mm) column and the fractions containing annexin V-N1 (detected by dot-blot) were pooled and

concentrated. The inhibitory activity of annexin V-N1 was determined by clotting activity as described above and the results shown that annexin V-N1 and the activity as described above and the results showed that annexin 5 V-N1 and the wild type annexin have similar activities. The protein sequence analysis confirmed that annexin V-N1 had the intended amino terminal oligopeptide with deletion of the amino terminal methionine.

10 All references cited herein are herein incorporated by reference in entirety.

It will be appreciated that the methods and compositions of the present invention are capable of being incorporated in the form of a variety of embodiments, only a few of which have been illustrated and described above. The invention may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than the foregoing description. All changes which come within the meaning and range of equivalency of the claims are to 20 be embraced within the scope of the invention.

We claim:

1. A conjugate comprising:

(a) a first compound having affinity for phospholipids, with a dissociation constant that is not greater than about 10^{-7} M; and

(b) a second compound that lyses thrombi or is a precursor of a compound that lyses thrombi.

2. The conjugate of claim 1 wherein said first compound is an annexin.

10 3. The conjugate of claim 2 wherein said annexin is annexin V.

4. The conjugate of claim 1 wherein said second compound is streptokinase, urokinase, tissue plasminogen activator or prourokinase.

15 5. The conjugate of claim 1 wherein said second compound is a modified-streptokinase, urokinase, tissue plasminogen activator or prourokinase.

6. The conjugate of claim 5 wherein a deletion type compound is the second compound.

20 7. The conjugate of claim 4 wherein said second compound is urokinase.

8. A therapeutic composition comprising:

(a) a first compound having affinity for phospholipids, with a dissociation constant that is not greater than about 10^{-7} M;

(b) a second compound that lyses thrombi or is a precursor of a compound that lyses thrombi; and

(c) a pharmaceutically acceptable carrier, diluent or excipient.

9. The therapeutic composition of claim 8 wherein said first compound is annexin.

5 10. The therapeutic composition of claim 9 wherein said first compound is annexin V.

11. The therapeutic composition of claim 8 wherein said second compound is streptokinase, urokinase, tissue plasminogen activator or prourokinase.

10 12. The therapeutic composition of claim 8 wherein said second compound is a modified streptokinase, urokinase, tissue plasminogen activator or prourokinase.

13. The therapeutic composition of claim 12 wherein a deletion type compound is the second compound.

15 14. The therapeutic composition of claim 11 wherein said second compound is urokinase.

15. A method of lysing thrombi comprising administering to a host in need of treatment a therapeutically effective amount of a composition comprising:

(1) a conjugate comprising:

(a) a first compound having affinity for phospholipids, with a binding constant that is not greater than about 10^{-7} M; and

25 (b) a second compound that lyses thrombi or is a precursor of a compound that lyses thrombi; and

(2) a pharmaceutically acceptable carrier, diluent or excipient.

16. The method of claim 15 wherein said first compound is an annexin.

17. The method of claim 16 wherein said first compound is annexin V.

5 18. The method of claim 15 wherein said second compound is streptokinase, urokinase, tissue plasminogen activator or prourokinase.

10 19. The method of claim 15 wherein said second compound is a modified streptokinase, urokinase, tissue plasminogen activator or prourokinase.

20. The method of claim 19 wherein a deletion type compound is the second compound.

21. The method of claim 18 wherein said second compound is urokinase.

15 22. The conjugate of claim 1 having been prepared by chemically coupling said first compound and said second compound.

20 23. The conjugate of claim 1 having been prepared by forming a fusion protein of said first compound linked to said second compound, wherein said fusion protein is expressed by a host carrying recombinant nucleic acids comprising coding sequences of said first and second compounds and recovering said fusion protein.

25 24. The conjugate of claim 5 wherein a hybrid type compound is the second compound.

25. The therapeutic composition of claim 12 wherein a hybrid type compound is the second compound.

26. The method of claim 19 wherein a hybrid type compound is the second compound.

27. A method of preparing a conjugate comprising:

5 (a) a first compound having affinity for phospholipids, with a dissociation constant that is not greater than about 10^{-7} M; and

10 (b) a second compound that lyses thrombi or is a precursor of a compound that lyses thrombi; which comprises chemically coupling said first compound and said second compound.

15 28. The method of claim 27, wherein an oligopeptide comprising a cysteine residue is attached to the amino terminus of said first compound and an SH group of a cysteine residue is chemically coupled with an NH₂ group of said second compound.

29. The method of claim 27, wherein said first compound comprises sulhydryl groups.

30. The method of claim 27, wherein said second compound comprises iodoacetyl groups.

20 31. A method of preparing a conjugate comprising:

25 (a) a first compound having affinity for phospholipids, with a dissociation constant that is not greater than about 10^{-7} M; and

(b) a second compound that lyses thrombi or is a precursor of a compound that lyses thrombi, which comprises:

(1) constructing a plasmid carrying a gene encoding the first compound and a gene encoding

the second compound directly or indirectly linked thereto,

5 (2) introducing the plasmid into a host to obtain a transformant,

10 (3) cultivating the transformant to allow it to express a fusion protein consisting of the first compound and the second compound directly or indirectly linked thereto, and

15 (4) recovering the fusion protein from the culture.

32. The method of claim 31, wherein the plasmid carries, in the 5' to 3' orientation, the gene encoding the second compound, a linker and the gene encoding the first compound.

15 33. The method of claim 32, wherein the linker has a base sequence encoding the 50th to 64th amino acid residues of the amino acid sequence of hirudin.

20 34. The method of claim 31, wherein the plasmid carries, in the 5' to 3' orientation, the gene encoding the first compound, a linker and the gene encoding the second compound.

1/3

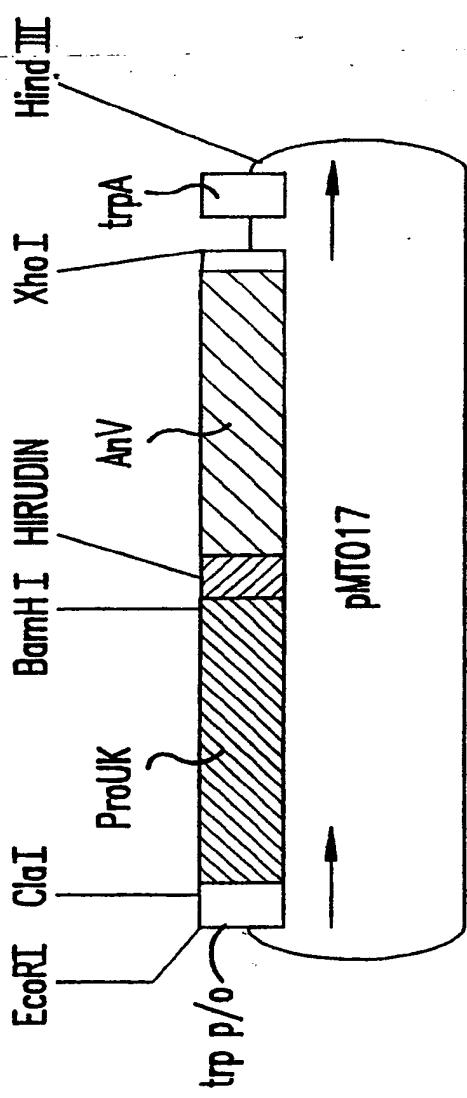


FIG. 1

2/3

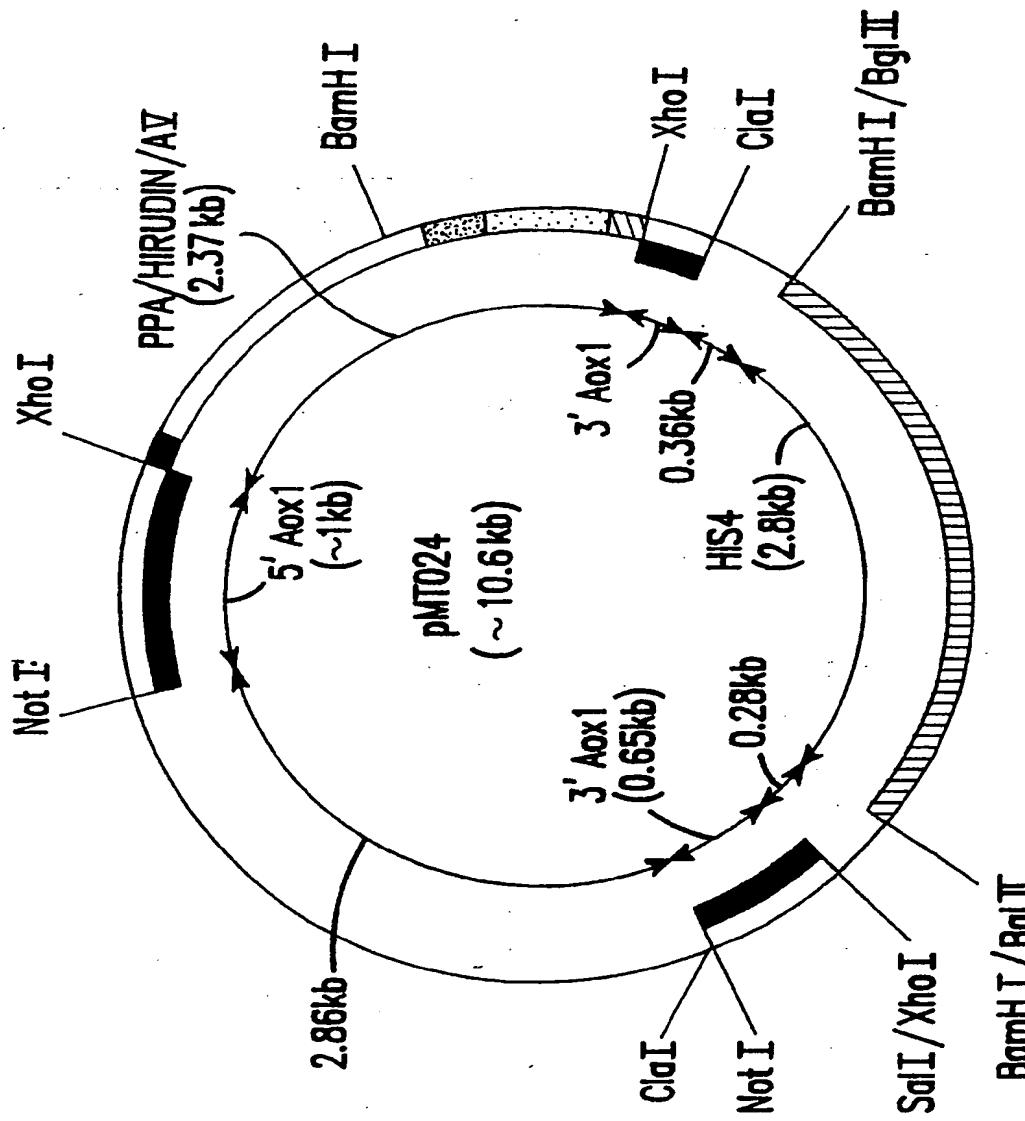


FIG. 2

3/3

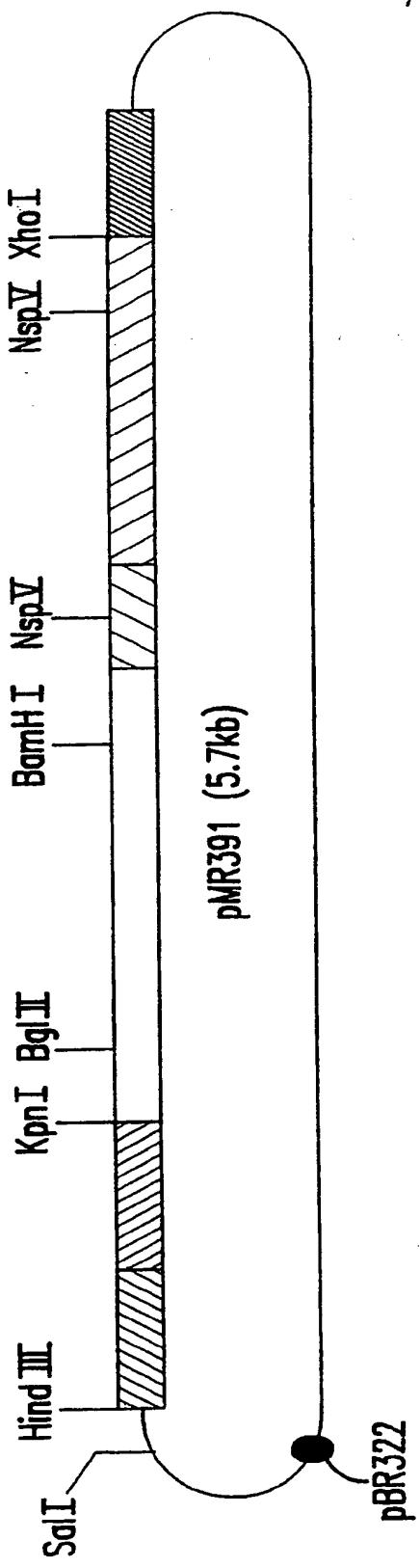


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/03960

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.2, 94.63, 94.64; 435/69.7, 172.3, 188, 215, 216, 240.2, 320.1; 514/2, 12, 21; 530/350, 402, 404, 405, 406; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

search terms: annexin, streptokinase, urokinase, prourokinase, tissue plasminogen activator, hybrid, chimeric, fusion, conjugate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences USA, Volume 84, issued October 1987, J.M. Schnee et al., "Construction and expression of a recombinant antibody-targeted plasminogen activator", pages 6904-6908, see abstract; "Materials and Methods"; Figures 2-4; page 6904, first column.	1-34
Y	Biochemistry, Volume 26, No. 25, issued 1987, T. Funakoshi et al., "Primary structure of human anticoagulant protein", pages 8087-8092, see abstract; "Materials and Methods"; Figure 4; page 8087, column 1.	1-34
Y	A. White et al., "Principles of Biochemistry", published 1978 by McGraw-Hill (N.Y.), see pages 916-928, especially Figures 29.5, 29.7 and pages 922 and 925.	1-34
Y	The Journal of Biological Chemistry, Volume 262, No. 24; issued 25 August 1987, L. Pierard et al., "Mutant and chimeric recombinant plasminogen activators. Production in eukaryotic cells and preliminary characterization", pages 11771-11778, see abstract; "Experimental Procedures"; Tables II-III.	5-7, 12-14, 19-21, 24-26

Further documents are listed in the continuation of Box C. See patent family annex.

• Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
• "A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
• "B" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
• "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
• "O" document referring to an oral disclosure, use, exhibition or other means		
• "P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

23 August 1992

Date of mailing of the international search report

25 AUG 1992

Name and mailing address of the ISA/
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

RICHARD C. EKSTROM



Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03960

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochemical and Biophysical Research Communications, Volume 130, No. 1, issued 16 July 1985, C.L. Sevilla et al., "Plasminogen activator-anti-human fibrinogen conjugate", pages 91-96, see abstract; "Materials and Methods."	1-22, 24-30
Y	Journal of Applied Biochemistry, Volume 6, issued 1984, S. Hashida et al., "More useful maleimide compounds for the conjugation of Fab' to horseradish peroxidase through thiol groups in the hinge", pages 56-63, see abstract; "Materials and Methods."	1-22, 24-30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03960

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 37/02, 37/54, 37/62; C07H 15/12, 17/00; C07K 3/06, 3/08, 13/00; C12N 5/00, 9/70, 9/72, 9/96, 15/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/94.2, 94.63, 94.64; 435/69.7, 172.3, 188, 215, 216, 240.2, 320.1; 514/2, 12, 21; 530/350, 402, 404, 405, 406;
536/27

THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)